

Thermophilic Methanogenesis in a Hot-Spring Algal-Bacterial Mat (71 to 30°C)

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Algal-bacterial mats which grow in the effluent channels of alkaline hot springs provided an environment suitable for studying natural thermophilic methane-producing bacteria. Methane was rapidly produced in cores taken from the mat and appeared to be an end product of decomposition of the algal-bacterial organic matter. Formaldehyde prevented production of methane. Initial methanogenic rate was lower and methanogenesis became exponential when samples were permitted to cool before laboratory incubation. Methanogenesis occurred and methanogenic bacteria were present over a range of 68 to 30°C, with optimum methanogenesis near 45°C. The temperature distribution of methanogenesis in the mat is discussed relative to published results on standing crop, primary production, and decomposition in the thermal gradient. The depth distribution of methanogenesis was similar to that of freshwater sediments, with a zone of intense methanogenesis near the mat surface. Methanogenesis in deeper mat layers was very low or undetectable despite large numbers of viable methanogenic bacteria and could not be stimulated by addition of anoxic source water, sulfide, or a macronutrient solution.

Interest in the conversion of organic wastes to methane has led to the consideration of thermophilic waste digestion (8, 13, 15). In such studies, wastes which obtain from environments of moderate temperature (e.g., cattle manure or domestic sludge) have been increased in temperature until the maximum conversion rate of waste to methane is reached. Among the advantages of thermophilic decomposition is the reduction of digester turnover time, presumably because the doubling time of methanogenic bacteria is shortened. This has resulted in greater methanogenic efficiency at elevated temperature.

A logical approach to studies on thermophilic decomposition is to perform such studies in natural high-temperature environments in which organic matter is being synthesized and decomposed. The effluent channels of many alkaline hot springs are inhabited by a unicellular blue-green alga (cyanobacterium) (*Synechococcus lividus*) and a filamentous photosynthetic bacterium (*Chloroflexus aurantiacus*), which take advantage of sunlight and elevated temperatures to produce an algal-bacterial mat at rates among the greatest known in nature (5). Recently, Doemel and Brock (10) have demonstrated that these algal-bacterial mats reach a steady state with respect to the synthesis and decomposition of organic matter in the mat. The algal-bacterial mat is present in the effluent channels of hot

springs over a temperature range of about 74 to 30°C, so that studies on decomposition can be performed over a natural temperature gradient in which microorganisms involved in anaerobic decomposition might have evolved at constant high temperature.

A methanogenic bacterium similar to *Methanobacterium thermoautotrophicum* (20) has been isolated from the algal-bacterial mat of Octopus Spring in Yellowstone National Park (19; J. G. Zeikus, University of Wisconsin, personal communication). However, no systematic studies of methanogenic bacterial activity have been performed in natural environments in which organic matter decomposes at high temperature. I report here on the distribution of methane-producing bacteria and their activity with respect to temperature and depth in an algal-bacterial mat of an alkaline hot-spring effluent.

MATERIALS AND METHODS

Study area. Studies were performed at Octopus Spring, an alkaline hot spring (pH 8.5), located about 0.15 km SSE of Great Fountain Geyser in the White Creek drainage in Yellowstone National Park. The thermal stability of this spring has been discussed by Doemel and Brock (10). Sampling was confined to algal-bacterial mats in the southernmost effluent channel, except for placement of a gas collector (see below) at 55°C along the southern shoulder of the spring. Temperature was measured with a mercury

thermometer. Temperatures were stable at given locations throughout this study so that sampling locations for given temperatures were always the same. pH was measured in the laboratory with a pH Master pH meter (VWR Scientific).

Sampling. Whole core samples were removed from the algal-bacterial mat with a no. 4 brass cork borer (50.3 mm²) and transferred directly to roller culture tubes (Bellco Glass, Inc.) or to 1-dram (3.9-g) glass vials (14.5 by 45 mm; Kimble), which were sealed in the field anaerobically (11; except that no copper reducing column was used in the field) under a stream of helium (Linde) with butyl rubber stoppers (A. H. Thomas, recessed butyl rubber stopper, size 00). Except in initial studies in which vials were returned to the laboratory at ambient temperature, samples were either immediately returned to the effluent channel for in situ incubation or placed in insulated containers which maintained temperature at within 10° of the in situ temperature and prevented light-stimulated O₂ production during transit to the laboratory (2 to 8 h). Due to changes in flow rate, the temperature over a given mat area varied naturally about 5 to 10°C. Formaldehyde (0.5 ml of a 40% solution) was added to inhibit biological methane production in nonbiological controls and to stop methanogenesis after in situ incubation. Samples incubated in situ were shielded from light by two layers of black plastic to prevent algal oxygen evolution which might inhibit methanogenesis. Immediately upon return to the laboratory, samples were placed in incubators at temperatures near the in situ temperature. Time courses began with removal of cores from the mat.

For depth profile studies, whole cores were removed with a sawed-off 3-ml plastic disposable syringe (Becton-Dickinson & Co.) so that cores were minimally exposed to air (same inside diameter as a no. 4 cork borer). Cores were subdivided with a scalpel between natural laminae (see figure legends for approximate interval lengths) as the core was extruded into vials gassing under helium. After being closed anaerobically (as described above), subcore samples were returned to a temperature near the in situ temperature. To prevent subcores from drying, 0.5 ml of anoxic, distilled water was injected. In some experiments, 0.5 ml of anoxic (i) Octopus Spring water, (ii) Octopus Spring water containing 0.03% (wt/vol) Na₂S·9H₂O (final concentration), or (iii) macronutrient solution providing 0.1% (wt/vol) NH₄Cl, 0.04% (wt/vol) K₂HPO₄·3H₂O, and 0.01% (wt/vol) MgCl₂·6H₂O (final concentrations) was added instead of distilled water. Solutions were made anoxic by boiling, cooling, and tubing under helium or nitrogen. In most-probable-number analysis, whole cores were subdivided in the laboratory. To avoid excessive exposure to oxygen during sampling while interval lengths were being measured, depths were recorded as grams (dry weight) determined after incubations. Because the depth intervals studied were above obvious bands of inorganic detritus, the assumption that the density of the mat is constant with depth is probably valid.

Gases emitted above the algal-bacterial mat were collected by pushing serum bottles (100 ml, with bottoms removed and stoppered with a recessed butyl

stopper; area, 576 mm²) into the mat to a depth of about 3 cm. Helium was used to displace air inside the serum bottles while the bottle was held beneath the surface of the water and before insertion into the mat. The gas headspace was approximately 60 ml. Duplicate samples of different volumes of gas were removed in helium-flushed syringes and transferred to helium-filled sample vials (as described above) for analysis in the laboratory.

Chemical analyses. Methane and hydrogen concentrations were determined using a Carle model 8500 gas chromatograph with a thermal conductivity detector and a stainless-steel column (OD, 23 feet by 1/8 inch [ca. 7 m by 3.18 mm]) packed with Porapak Q (60/80 mesh; Carle Inst. Co.) with helium carrier flow at 43 ml/min and isothermal oven control at 30°C. Peak height was a linear function of concentration over the ranges analyzed. Gas subsamples were removed with a gas-tight syringe. Concentrations were corrected to standard temperature and pressure and are presented here as the total amount of methane or hydrogen in the gas headspace of a given sample. Because mat thickness was variable and most methanogenesis occurred near the surface of the mat (see below), methanogenesis in whole cores is reported per core (50.3 mm²), rather than by another normalizing factor, such as dry weight.

Volatile fatty acids were determined by using a Beckman GC 5 gas chromatograph with a flame ionization detector and a stainless-steel column (OD, 6 feet by 1/8 inch [ca. 1.83 m by 3.18 mm]) packed with Chromosorb 101 (Supelco) with on-column injection, helium carrier flow of 97 ml/min, and isothermal oven, injector, line, and detector controls at 160°C. Peak area was a linear function of acetic acid concentration over a range of 0 to 100 nmol, and the smallest detectable amount of acetic acid was about 1 nmol.

Sulfate was determined by turbidimetric assay (1).

Enumeration of methanogenic bacteria. The most probable number of methane-producing bacteria was estimated by using a three-tube assay (1), standard anaerobic technique (11), and a medium designed to provide a complete nutritional environment and H₂ as an electron donor common to all known methanogenic bacterial isolates (6, 12, 18, 19). The medium, consisting of 1.0 g of NH₄Cl, 0.4 g of K₂HPO₄·3H₂O, 0.1 g of MgCl₂·6H₂O, 2.0 g of Trypticase (BBL), 2.0 g of yeast extract (Difco), 1.5 g of NaHCO₃, 0.5 g of cysteine·HCl, 0.001 g of resazurin, and 333 ml of water from Octopus Spring, was made to 1 liter with distilled water, adjusted to pH 6.6, boiled under N₂, and tubed under H₂. After autoclaving, a sterile anoxic solution of Na₂S·9H₂O (3% [wt/vol] in distilled water) was diluted 1:100 in the tubed medium to give a final concentration of 0.3 g of Na₂S·9H₂O per liter and a final pH of 7. Samples were transferred under H₂ by the open-tube method (11) to an initial dilution tube containing the same medium and glass beads, and the sample was dispersed by vigorous shaking. It was impossible to completely disperse the algal-bacterial mat so that the numbers of bacteria reported here most likely underestimate the true population density. Subsequent dilutions were made by the closed-tube method (by syringe; 11). Medium was warmed to the

temperature of the samples before inoculation. After 2 weeks of incubation at the in situ temperature, tubes were analyzed for methane in the gas phase. Further incubation did not alter the results.

RESULTS

Chemistry of Octopus Spring water. Concentrations of various chemical constituents of Octopus Spring water have been previously reported (9). Sulfate levels were found to be 16.6 mg/liter in the present study. No volatile fatty acids were detected in 10- μ l samples of water collected from the effluent channel where the algal-bacterial mat was growing. Since the limit of detection for acetic acid was about 1 nmol, the maximum concentration of low-molecular-weight organic acids present was calculated to be 0.1 μ mol/ml. No methane or hydrogen was detected in the gas phase above water samples incubated under helium.

Biological methanogenesis in the algal-bacterial mat. In initial experiments, cores of the algal-bacterial mat were removed at locations along the effluent channel where average temperatures were 45, 55, and 65°C. The cores were placed immediately into tubes under helium and returned to the laboratory at ambient temperature (ca. 20°C for 5 to 8 h) for incubation at temperatures near the in situ temperature. Methane production was initially slow and appeared to increase exponentially with incubation (Fig. 1A). More rapid methanogenesis was observed at 55°C than at 45°C. No methane was produced at 65°C; however, hydrogen accumulated in the gas phase. The addition of formaldehyde prevented methanogenesis, indicating a biological origin of methane in the gas phase.

When cores were maintained between sampling and laboratory incubation within 10°C of the temperature of the mat from which they came, methanogenesis occurred at much greater initial rates and appeared linear during the first few days of incubation (Fig. 1B). Because the differences in the kinetics of methane production due to exposure to temperatures below the normal mat temperature were suggestive of major reductions in population density and subsequent exponential growth, methanogenesis in cores not exposed to temperature reduction was considered a more valid estimation of the activity of indigenous methanogenic populations and, in all subsequent experiments, samples were either returned to the laboratory at near in situ temperature or incubated in situ. Interestingly, the initial rate of methanogenesis in cores protected from large variations in temperature was more rapid at 45°C than at 50°C.

Methanogenesis along the thermal gra-

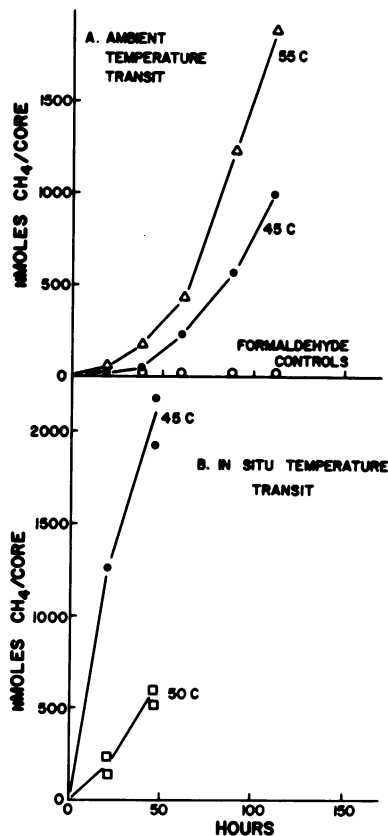


FIG. 1. Time courses for methane production in whole cores (50.3 mm²) from areas of the Octopus Spring algal-bacterial mat in which temperature was 45°C (●), 50°C (□), or 55°C (Δ). Formaldehyde controls (○) contained 0.5 ml of Formalin. No methane was produced in cores taken from a 65°C region of the mat. Temperature during transit was either (A) ambient or (B) within 10°C of the in situ temperature. Laboratory incubation was at the in situ temperature.

dient: (i) methane production in laboratory incubation. Core samples were taken at locations where the average mat temperature was 40, 45, 50, 55, 60, or 65°C and incubated in the laboratory at corresponding temperatures. Initial methanogenesis was defined as the amount of methane produced per core at the time of the first gas analysis (21 h) and is plotted as a function of temperature in Fig. 2A. At 40, 45, and 50°C, methanogenesis was linear up to 200 h so that the initial methanogenesis could be used to calculate methanogenic rate. Maximum initial methanogenesis occurred at 45°C, with sharp reductions above and below this temperature. Further incubation of samples at 55 and 60°C resulted in exponential increases in methane production and suggested that, although

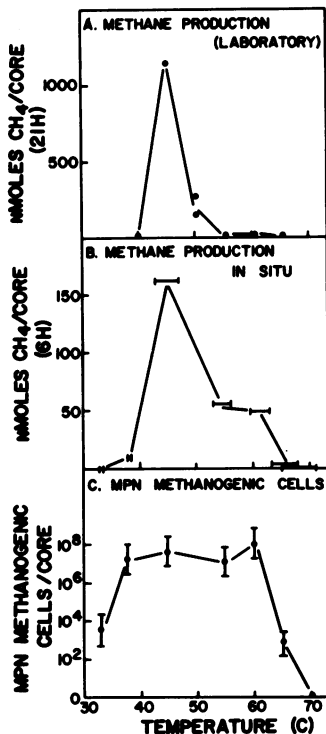


FIG. 2. (A) Methane produced in a 21-h incubation of whole cores (50.3 mm²) collected from the Octopus Spring algal-bacterial mat at various temperatures and incubated at the same temperatures in the laboratory. Transit temperature was within 10°C of the in situ temperature. (B) Methane produced in a 6-h in situ incubation of whole cores collected at various mat temperatures. Bars indicate in situ temperature range during incubation. (C) Most probable number (MPN) of methanogenic bacteria in whole cores taken from various temperatures in the mat. Bars indicate 95% confidence limits.

initial rates of methanogenesis were low, enrichment could occur rapidly. Small amounts of methane were produced at 40°C on extended incubation (120 h). At 65°C, hydrogen was produced and no methane could be detected.

(ii) **Methane production in in situ incubation.** To minimize temperature alterations, samples were removed from the mat, placed under helium, and incubated in darkened vials for 6 h in the effluent channel. Samples were poisoned by addition of formaldehyde and the gas phase was analyzed for methane in the laboratory (Fig. 2B). Maximum methanogenesis occurred in the 43 to 47°C range. Methanogenesis was significantly less at lower temperatures. At 53 to 57°C and 59 to 63°C, methanogenesis was about one-third that at 43 to 47°C. At 63 to 68°C, very little methanogenesis was observed. No detectable methanogenesis occurred in sam-

ples incubated at a range of 65 to 71°C.

(iii) **Gas released above the algal-bacterial mat.** Gas emitted above the mat was collected in serum bottles placed over areas in which the average temperature was 45, 55, or 65°C. More rapid accumulation of methane occurred in the collector placed above the mat at 45°C than at 55°C (Fig. 3). Neither methane nor hydrogen was detected in the collector placed above the mat at 65°C.

(iv) **Methanogenic bacteria along the temperature gradient.** The most probable numbers of methane-producing bacteria (which developed at temperatures corresponding to in situ temperatures) in cores taken from those areas of the mat in which the average temperature was 33, 38, 45, 55, 60, 65, or 70°C are graphed in Fig. 2C. Although the distribution of methanogenic bacteria per core was similar to the distribution of methanogenic activity over the temperature gradient, it is interesting to note large populations in areas in which methanogenesis was relatively low (at 38, 55 or 60°C).

Methanogenesis with depth in the mat. Core samples collected at an average mat temperature of 45°C were subdivided and incubated for analysis of gas production. Figure 4 shows the time courses of methane production in mat subcores. In the top mat layer, rapid methanogenesis occurred only after a lag period and

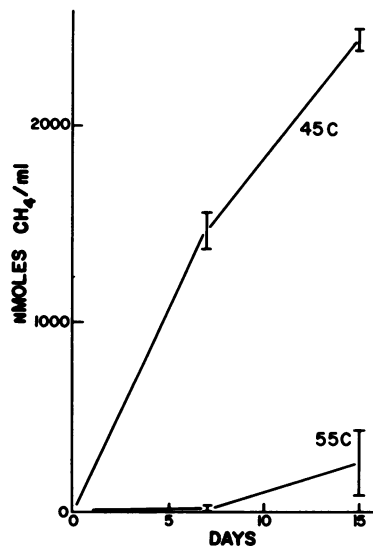


FIG. 3. Methane collected in serum bottles (576 mm²) placed above the Octopus Spring algal-bacterial mat at 45 and 55°C. No methane was detected in gas collected above a 65°C region of the mat. Bars indicate the range of duplicate gas samples. No methane was detected immediately after placement of the collectors.

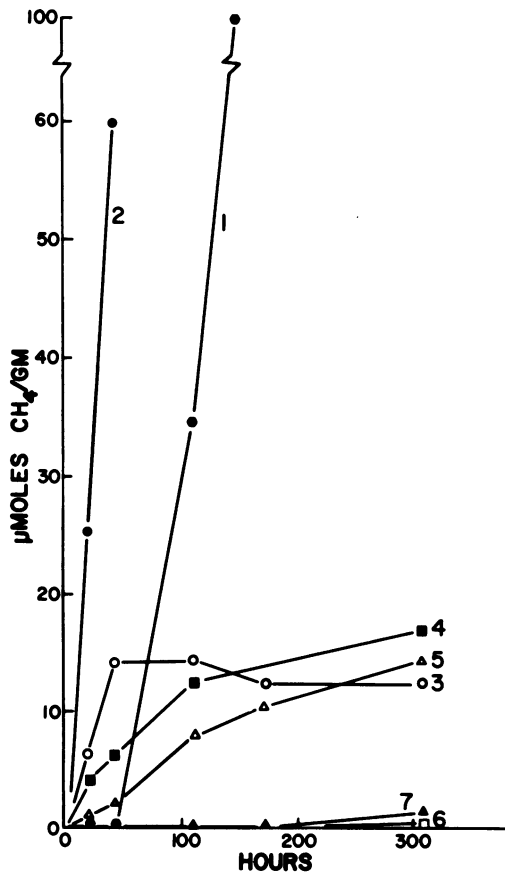


FIG. 4. Time courses for methane production in subcores of a whole core (50.3 mm^2) taken from a 45°C region of the Octopus Spring algal-bacterial mat. Layers are numbered consecutively, top to bottom as indicated. Approximate intervals for subcores were as follows: layers 1 to 6, 1 mm; layer 7 (bottom), 1 cm. Approximate total length, 1.6 cm.

appeared to be exponential, suggesting enrichment of methanogenic bacteria whose initial activity was undetectable. This is not surprising, considering the probable production of oxygen by *S. lividus* in this layer (10). With incubation in the dark, photosynthesis is arrested and methanogenic bacteria can become enriched. In all other mat subcores, methanogenesis appeared linear with time. Maximum methanogenesis was observed in the layer just adjacent to the top layer. In deeper layers, the rate of methane production was lower with depth until methanogenic activity became almost undetectable. In layers adjacent to the layer of maximum methanogenesis, time courses indicated that methanogenesis became limited after about 50 h.

When the initial rate of methanogenesis is plotted as function of length (Fig. 5A), it is clear

that most of the methanogenic activity was located in a thin zone just below the top mat layer. With increasing mat depth, methanogenic activity decreased rapidly. Surprisingly, the most probable number of methanogenic bacteria was relatively constant with depth in the mat at 45°C (Fig. 5B), suggesting that the specific activity in methanogenesis per viable cell was reduced. Because the 95% confidence limits (not shown in Fig. 5B) overlapped from sample to sample, variations in most probable number with depth were considered within the error of the method.

The depth distribution of methane production at 55°C or of hydrogen production at 65°C was similar to that at 45°C (Fig. 6). In subcores from a mat sample taken at 55°C , time courses of methane production appeared exponential with low initial activity (as in Fig. 1A). However, after 174 h of incubation, methanogenesis appeared to have begun in the uppermost subcores and showed the depth distribution plotted in Fig. 6B. In subcores of a mat sample collected at 65°C , hydrogen production was linear with time in the uppermost mat layers and became limited after about 50 h (as in Fig. 4, layers 3 to 5). Again, the depth distribution (Fig. 6C) indicated that most hydrogen production occurred near the mat surface.

The addition of (i) anoxic Octopus Spring water, (ii) anoxic Octopus Spring water containing sulfide as a reducing agent, or (iii) an anoxic macronutrient solution (containing nitrogen and phosphorus) did not increase methanogenesis in deeper layers of a core taken from 45°C , suggesting that improper redox level and insufficient supply of inorganic macronutrients probably do not limit methanogenic activity in mat subcores below the zone of maximum methanogenesis. No additions caused inhibition of methanogenesis in upper mat layers. However, the addition of the mineral salts solution resulted in stimulation of methanogenesis in the upper mat layers, suggesting that methanogenesis may be limited by insufficient amounts of inorganic macronutrients supplied to the mat from the spring-water.

DISCUSSION

Rapid methanogenesis occurred in association with organic mats which developed as the result of algal and bacterial photosynthesis in the effluent channel of an alkaline hot spring. Because no methanogenic substrates (hydrogen, formic, or acetic acid) were detected in the springwaters that bathe the mats, it is assumed that methane was produced as an end product of an anaerobic food chain involved in decomposition of the

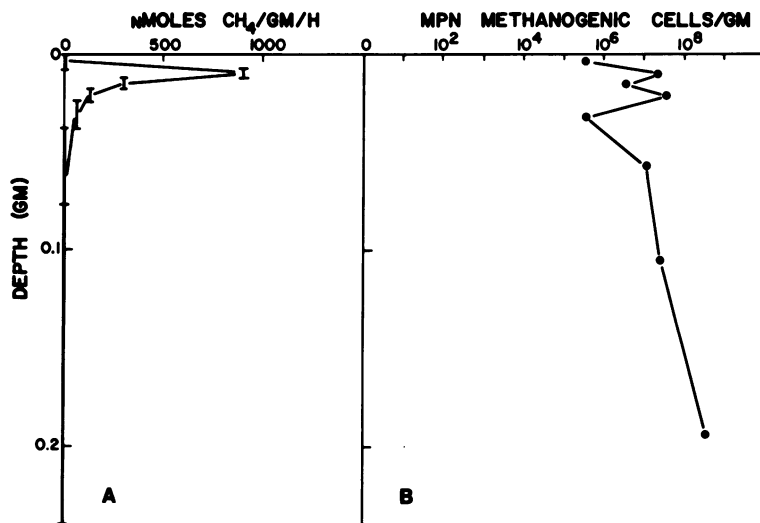


FIG. 5. Depth distribution of initial methane production rate (A) and most probable number (MPN) of methanogenic bacteria (B) in subcores of a whole core (50.3 mm^2) taken from a 45°C region of the Octopus Spring algal-bacterial mat. Bars indicate depth interval. (A) Depth intervals in approximate lengths are given in the legend to Fig. 4. (B) Depth intervals in approximate lengths (data points are at the midpoint of the interval) are as follows (top to bottom): 1, 1, 1.5, 1, 1, 2, 3, and 5 mm. See text for explanation of the use of grams as a measure of depth.

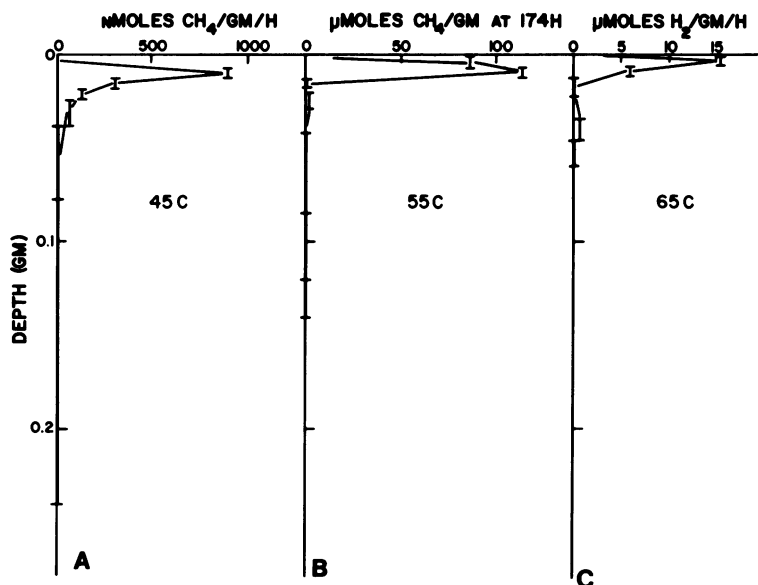


FIG. 6. Depth profile of initial methane production rate at 45°C , methane produced after 174 h of incubation at 55°C , and initial hydrogen production rate at 65°C , in subcores of whole cores (50.3 mm^2) of the Octopus Spring algal-bacterial mat taken from 45, 55, and 65°C regions of the mat, respectively. Approximate depth intervals in length are as follows (top to bottom): (A) same as for Fig. 4 and 5; (B) 2, 1, 1.5, 3, 1, 1, 2, 1, and 1 mm; approximate total length, 1.35 cm; (C) 0.5, 1, 1, 1, 1, and 2 mm; approximate total length, 0.65 cm. Bars indicate depth interval. See text for explanation of the use of grams as a measure of depth.

algal-bacterial organic matter.

A blue-green alga (cyanobacterium) (*S. lividus*) and a photosynthetic bacterium (*C. aurantiacus*) are responsible for mat synthesis, but

self-shading restricts mat synthesis to the uppermost mat layers (2, 4). As organic matter is buried by new mat growth, protein components and mat thickness decrease with rapid initial

decomposition, and there are significantly slower rates of decomposition with deeper burial (10). The production of H_2S in 55°C Octopus Spring mats (21) and the distribution of methanogenesis (and hydrogen production at 65°C) found in the present study also suggest a zone of intense decomposition immediately adjacent to the growing mat surface, despite the proximity of O_2 -producing blue-green algae. Apparently, decomposition becomes limited only a few millimeters below the mat surface, due to factors other than poor macronutrient supply. The remaining deeper layers of the mat (ca. 1 to 3 cm depending on temperature) are presumably comprised of organic matter which is either decomposed more slowly or resists further anaerobic decomposition.

The depth distribution of methanogenesis in the high-temperature algal-bacterial mat is similar to the distribution of methanogenesis in freshwater sediments (12). Although the potential for sulfate reduction has been demonstrated in Octopus Spring mats (9), the sulfate level in Octopus Spring water is low (about 17 mg/liter) and sulfate reduction does not appear to restrict methanogenesis in upper layers of the mat as has been hypothesized in other freshwater sediments (7, 17). In high-sulfate environments such as oceanic sediments, the mutual exclusion of sulfate and methane has been repeatedly observed (12, 14). Whether large amounts of organic matter may be buried more rapidly than they are decomposed in sedimentary environments is an interesting question, especially relative to subsequent chemical diagenesis of sediment organic matter which might occur with even greater burial in conventional aquatic environments. If algal-bacterial mats of siliceous alkaline hot springs are modern day equivalents of stromatolytic microflora of siliceous rocks (10), then the nature of organic matter which resists anaerobic decomposition in these mats is also important to the interpretation of the morphology and organic geochemistry of microfossils.

Since the algal-bacterial mat occupies a natural temperature gradient, it was possible to observe the effect of temperature on methane production. Although increased methanogenesis with increasing temperature might be expected based on experiments on thermophilic digestors (8, 13, 15), maximum methanogenesis was found at a temperature near 45°C along the thermal gradient. There is reasonable agreement in both the qualitative (Fig. 2A, 2B, and 3) and quantitative (Table 1) estimation of methanogenesis among three different experimental approaches to relate methane production to temperature. It is interesting that a significant amount of the methane produced in the mat was released to

the gas phase above the mat.

In earlier studies, primary production by photosynthetic algae and bacteria was found to be optimal between 48.3 and 58.5°C, although standing crop was maximal between 55 and 60°C (3). Inherent thermal limitations were thought to limit both primary productivity and standing crop at temperatures greater than 60°C (5). At temperatures below 40°C, the grazing of brine flies reduces standing crop (16). Between 40 and 48.3°C, an anomaly between primary productivity and standing crop has existed (5). Decreases in protein and in thickness with depth suggested a temperature optimum for anaerobic decomposition between 52 and 56°C (10), but few data were taken in the 40 to 50°C range. Since methanogenesis is an index of complete anaerobic decomposition, the temperature distribution of methanogenesis may be a more precise indication of the temperature distribution for anaerobic decomposition of the mat. The standing crop between 45 and 55°C would thus be lower than at the temperature optimum for standing crop (55 to 60°C) where primary productivity was optimal but anaerobic decomposition suboptimal.

The direct influence of temperature on the activity of methanogenic bacteria is difficult to resolve because methanogenic bacteria occupy a terminal niche in anaerobic decomposition and may be influenced by other environmental parameters that change as a result of temperature. Below 40°C, the perforation of the algal-bacterial mat by brine fly larvae (16) may create an environment that is too oxidizing for substantial methanogenesis. The reduction of methanogenesis at temperatures above the optimum (45°C) may be due to inherent thermal limitations of methanogens or to other ecological factors, causing reduction in the availability of methanogenic substrates. The rapid exponential increase in methanogenesis upon continued incubation of samples collected and incubated at 55 and 65°C lends support to the latter hypothesis. Since the potential for methanogenesis near the upper temperature extreme appears greater than that observed, it seems inappropriate to directly compare methanogenic rates in this thermal envi-

TABLE 1. Comparison of methanogenic rates at 45 and 55°C measured by various methods

Expt	Methanogenic rate (nmol/h per mm ²) at:	
	45°C	55°C
Laboratory incubation of cores	1.2	0.095
In situ incubation of cores	0.54	0.166
In situ gas collectors	0.73	0.089

ronment to rates found in other natural or digester environments.

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